TITLE: The spatiotemporal regulation of RAS signalling

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ABSTRACT: Nearly 30% of human tumours harbour mutations in RAS family members. Post-translational modifications and the localization of RAS within subcellular compartments affects RAS interactions with regulator, effector and scaffolding proteins. New insights into the control of spatiotemporal RAS signalling reveal that activation kinetics and subcellular compartmentalisation are tightly coupled to the generation of specific biological outcomes. Computational modelling can help utilising these insights for the identification of new targets and design of new therapeutic approaches.

KEY WORDS: RAS, localization, site-specific signalling
ABBREVIATIONS: GEF; Guanine Exchange Factor, GAP; GTPase Activating Protein, GTP; Guanosine TriPhosphate, GDP; Guanosine DiPhosphate, PTM; Post-Translational Modification, HVR; HyperVariable Region, ER; Endoplasmic Reticulum, PM; Plasma Membrane

The RAS protein family and posttranslational processing

Since H-RAS was discovered as an oncogene more than 30 years ago [1, 2] there has been constant interest to understand the regulation and functions of the RAS family members. Nearly 30% of human tumours present a mutation in RAS genes [3, 4]. The *RAS* family comprises 3 genes that encode 4 isoforms: *H-RAS, N-RAS, K-RAS4A* and *K-RAS4B* (hereafter referred as *K-RAS*) [5]. *K-RAS* is the most frequently mutated isoform in human tumours, while mutations in *H-RAS* are the rarest [6]. RAS proteins are small GTPases that function as molecular switches depending on the guanine nucleotide that is bound to RAS. For RAS activation, GTP loading is required. This reaction is catalysed by Guanine Exchange Factors (GEFs), which stimulate the release of GDP and the subsequent binding of GTP, which is >10fold more abundant. GTP binding evokes a conformational change [7], permitting RAS to interact with different effectors and signal through different networks. RAS proteins are deactivated by their intrinsic GTPase activity but this reaction is very slow. GTPase Activating Proteins (GAPs) accelerate GTP hydrolysis to GDP returning RAS to its inactive state.

RAS proteins undergo complex post-translational modifications (PTMs) and trafficking through different organelles targeting them to several cellular membranes including the endoplasmic reticulum (ER), Golgi complex, mitochondria, endosomes and plasma membrane (PM) (Figure 1). RAS processing modifies the CAAX box in the C-terminal hypervariable region (HVR), where RAS isoforms diverge in sequence. Briefly, RAS modification

begins with the farnesylation of the CAAX cysteine C186 catalysed by specific farnesyltransferases [8, 9]. This irreversible reaction allows the weak interaction of RAS with the ER membrane, and is followed by proteolysis of the final AAX mediated by RAS converting enzyme (Rce1) [10]. The next step is further methylation of the farnesylated cysteine by an isoprenylcysteine carboxymethyltransferase (Icmt) [11]. These PTMs are shared by different RAS isoforms. However, all RAS proteins require a second signal for stable binding to membranes. In the case of K-RAS the second signal is provided by a stretch of lysines in the HVR which due to their positive charge promote electrostatic interactions with negatively charged membrane phospholipids. In the case of N-RAS and H-RAS, the second signal is the palmitoylation of one or two cysteines in the HVR, respectively. This reversible reaction is catalysed by isoform specific palmitoyl acyltransferases (PATs) at the Golgi [12]. Together with the previous farnesylation this modification produces mature RAS with stable but reversible membrane binding [13]. Importantly, in contrast to farnesylation, palmitoylation can be reverted by depalmitoylases. The depalmitoylation occurs at the PM and causes the release of RAS from the membrane to the cytosol and further diffusion to Golgi complex, where it can be re-palmitoylated [14]. This acylation/deacylation cycle is a mechanism that can determine some of the features of RAS activity and localization including trafficking between PM and Golgi complex, and between different PM microdomains [15]. It also may contribute to localisation specific signalling by exposing cycling RAS to compartment specific regulators and effectors.

Where and how RAS proteins signal

RAS proteins act as molecular switches that in their active GTP bound state can associate with a wide range of effector proteins through a shared, structurally highly conserved interface [16]. However, subtle differences in affinities give rise to a hierarchy of effector binding, which interestingly, is modified by the presence of oncogenic mutations that render RAS constitutively active [17]. Combined with different abundances of effector proteins in different cells and subcellular compartments, a large and varied assortment of RAS-effector complexes can be generated to enhance the functional diversity of RAS signalling. However, there is another twist to further diversify the RAS signalling repertoire.

RAS binding to cell membranes is essential for activation by specific GEFs upon cellular stimulation. Initially, it was thought that RAS could only signal from the PM. After the discovery of the existence of different PM subdomains several groups showed that the RAS isoforms localise to different PM domains and that they move between domains upon stimulation [18]. At that time it was also thought that RAS localisation at endomembranes was only related to RAS trafficking from ER to PM, as part of its posttranslational processing [19]. Nowadays, it is well recognised that RAS can also signal from endomembranes such as ER, Golgi or mitochondria [20-22]. Importantly, RAS isoforms are located within different cellular compartments as determined by the distinct PTMs of the HVR [23]. Moreover, the subcellular distribution of a given RAS isoform can be cell type specific [15].

Recently, phosphorylation and ubiquitination were discovered to impact the subcellular localisation of mature RAS. Phosphorylation of K-RAS on S181 by PKC re-locates K-RAS from the PM to the mitochondria where it binds to Bcl-XL to cause apoptosis [21, 24]. All RAS isoforms can be ubiquitinated. Polyubiquitination is stimulated by Wnt signaling and triggers RAS degradation, mediating a negative cross-regulation of the ERK Pathway by Wnt activation [25]. By contrast, mono- or di-ubiquitination regulates RAS localisation and function [26, 27]. H-RAS and N-RAS are mono-/di-ubiquitinated by the E3 ligase Rabex-5, which is localised at endosomes [28], resulting in accumulation of RAS proteins on endosomes and a concomitant decrease in ERK activation [26]. This modification also affects the biochemical function of RAS proteins, enhancing GTP loading of H-RAS, while impairing GTP hydrolysis by K-RAS [29]. Both changes promote activation. However, they also may re-programme downstream signalling by changing the affinity for specific effectors, e.g. Raf1 or PI3K [27]. Functional changes induced by phosphorylation and ubiquitination generate a rich repertoire of dynamic behaviour that can pattern biological responses [30].

Currently, the concept that RAS can signal from different subcellular membranes is well accepted, but for a long time it was thought that RAS proteins were only functional at the PM [31]. This dogma unravelled after the observation that GEFs are located at distinct endomembranes and may activate RAS in non-PM localizations. This hypothesis was confirmed when RAS-GRP was found to activate H-RAS and N-RAS at the Golgi [22, 32]. Subsequently, RAS-GRF1, RAS-GRF2 and SOS were described to activate RAS at the ER [33]. The idea of site-specific signalling of RAS was reinforced when GAP proteins were identified at endomembranes and different plasma membrane lipid domains. For example, CAPRI is located at the PM functioning as RAS-GAP in response to calcium [22]. Similarly, AnnexinA6 resides at the PM where it forms a complex with p120-GAP to selectively interact with and inactivate RAS in non-lipid raft microdomains and endosomes derived from the PM [34].

Specification of RAS signalling by scaffolds and spatiotemporal kinetics

Several groups showed that RAS regulates different signals from different locations. Using constitutively active RAS mutants targeted to specific sites, Philip's and Crespo's groups discovered that the activation of downstream signalling pathways occurs in a location-dependent manner [20, 35]. This was due to activation *in situ* at different subcellular sites rather than to transport of activated RAS from the PM to endomembranes. The requirement for Src kinase activity for RAS activation at endomembranes, but not at the PM, suggested that different mechanisms regulate RAS activation at distinct subcellular sites. Indeed, Src activates Phospholipase-Cγ1 stimulating the translocation of the RAS GEF RASGRP1 to the Golgi to activate RAS, whereas the same stimulus curtails RAS activation at the PM through the RAS-GAP CAPRI [22]. Intriguingly, the transient activation of RAS at the PM versus a slower but sustained activation at the Golgi determines negative versus positive T-cell selection in response to T-cell receptor (TCR) stimulation, respectively [36]. Although this view was challenged by observations that the TCR activates RAS exclusively at the PM [37], evidence from other cell types shows not only site specific RAS activation, but also coupling to different effector pathways from

different localisations. RAS effectors such as ERK, JNK, PI3K or RalGDS were differently regulated depending on RAS localization, e.g. RAS activated RalGDS preferentially from the Golgi and JNK from the ER [20, 35]. Moreover, cell proliferation and transformation were stimulated by RAS signalling from the PM and ER, but not the Golgi [20, 35]. Site-specific RAS signalling differentially regulated gene expression revealing that the largest transcriptional changes were induced by ER resident RAS, while RAS signalling from the disordered PM regulated the most site-specific genes [38]. Importantly, the site specific transcriptional changes were largely consistent with the biological functions observed.

In 2009, Casar et al. reported that RAS localization could affect the substrate choice by the RAS effector kinase ERK [39]. This phenomenon is based on the site specific presence of scaffold proteins. RAS signalling from lipid rafts can employ two different scaffold proteins, KSR1 and IQGAP, to activate the RAF-MEK-ERK pathway leading to the phosphorylation of either cPLA2 or EGFR respectively. However, when signalling from the ER RAS uses the scaffold Sef-1 to activate cPLA2. These data suggest that, by usage of intermediate proteins, site-specific RAS signalling can lead to the activation of distinct final effectors.

Moreover, the differential usage of effectors can provide both spatial and temporal organisation. The kinetic of activation of RAS from endomembranes is delayed and more sustained than activation from PM where it is transient and rapid [20, 40, 41]. ERK pathway activation from the PM is transient, while once RAS has trafficked to endosomes ERK activation becomes more sustained [26, 41, 42]. The physiological relevance of this temporal control of ERK signalling is evident from its link to cell fate decisions. The classical example are PC12 cells, which undergo proliferation in response to a transient ERK activation by EGF, or neuronal differentiation when ERK activity is sustained by NGF treatment [43]. Similarly, EGF driven proliferation of MCF7 mammary epithelial cells correlates with transient RAS and ERK activation, while HGF treatment causes differentiation and sustained RAS and ERK activity [44]. Both proliferation and differentiation require RAS signalling from the disordered PM [44]. Interestingly, EGF driven proliferation can be converted into differentiation by overexpressing H-RAS, which prolongs ERK signalling. Intriguingly, this conversion also requires H-RAS to signal form the disordered PM, whereas targeting H-RAS to the Golgi triggers apoptosis.

The control of RAS signalling by spatiotemporal mechanisms seems evolutionary conserved. The single RAS homologue of fission yeast supports activation of the mating pathway from the cell membrane, but morphology changes from endomembranes [45]. Taken together, these data clearly indicate that the compartmentalization of RAS signal is a regulatory mechanism that can determine cell fate decisions by generating differential biochemical pathway activation patterns. However, many of the fundamental mechanisms have been elucidated in cell lines and model systems using engineered and exogenously expressed proteins, and are not yet confirmed in higher organisms.

RAS localisation as therapeutic target

The high frequency of activating RAS mutations in cancer has spawned major efforts to inhibit mutated RAS or RAS signalling. A string of failures has earned RAS a reputation as being undruggable. The initial hope, based on successful animal studies, that farnesyltransferases (FTase) inhibitors,could antagonise RAS transformation was thoroughly dashed in clinical studies, as FTase inhibition is compensated by alternative geranylation [46]. The logic of inhibiting both types of transferases also disappointed as such compounds were unable to inhibit oncogenic K-RAS [47], which is the predominantly mutated RAS isoform in human tumours

Recent efforts targeting RAS subcellular localisation or interaction with effectors have rekindled cautious hope. Depalmitoylase inhibitors perturb the acylation/de-acylation cycle that assures precise RAS localisation reverting RAS transformation in cellular models [48]. Inhibition of farnesyl binding by PDEδ led to K-RAS spatial mis-organization and reversion of K-RAS transformation in cellular models [49, 50] Likewise, small molecule inhibitors that prevent the interaction of RAS with its effectors showing promise as a new paradigm for suppressing RAS transformation [51-53].

Systems Biology for the understanding of RAS networks

The conceptual simplicity of RAS functioning as a signalling switch betrays the diversity and complexity of its regulation and effector functions. Three decades of intensive research have revealed that manipulating this switch requires an understanding of its exquisite spatiotemporal regulation that is unlikely to be achievable by experimentation alone. Computational modelling of RAS regulation and signalling can reveal not only fundamental principles of RAS function, but importantly also into the most promising targets for interference. For instance, mathematical modelling was instrumental to understand the non-equilibrium processes that govern the formation of K-Ras nanoclusters in the PM during RAS activation [54]. Modelling the molecular mechanisms underlying the crosstalk between the EGFR and insulin receptor identified the adapter protein GAB1 as critical coincidence detector and signal amplifier that enhances EGFR mediated RAS signalling [55]. The power of mathematical models to identify pathway control nodes also lends itself to discover new drug targets or new, often non-obvious drug combinations. A main RAS effector pathway consists of an amplifying kinase cascade (RAF-MEK-ERK) with a negative feedback from ERK to RAF and RAS, which makes the pathways resistant to amplifier perturbations, i.e. MEK inhibitors. However, modelling shows that the concomitant weakening of the feedback that can be achieved by RAF inhibition can effectively block ERK activation [56]. This synergistic effect can suppress oncogenic RAS signalling and transformation [57]. A synergism between drugs that target two consecutive nodes in a linear pathway is highly unexpected but was clearly revealed by modelling.

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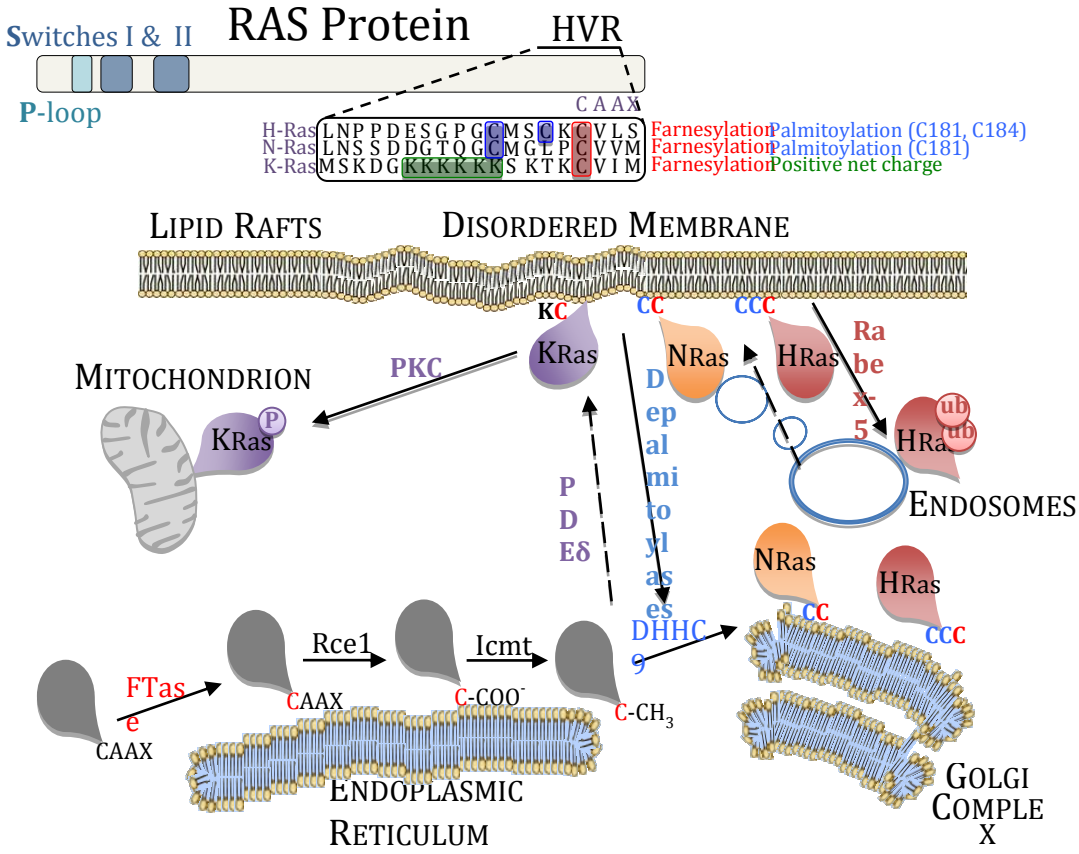

Figure 1. Ras maturation involves a series of posttranslational protein modifications (PTMs) and subcellular localisation changes. Ras family members differ in the C-terminal region which includes the CAAX box. This motif is the target of PTMs that allow the correct localisation of Ras at plasma- and endomembranes, and mitochondria. All three Ras isoforms are farnesylated by Farnesyl-transferase (FTase, red) inducing binding to the endoplasmic reticulum. There, Ras undergoes removal of the last three amino acids by Ras Converting CAAX Endopeptidase 1 (Rce1) and carboxymethylation by Isoprenylcysteine Carboxyl Methyltransferase (Icmt). For more stable membrane binding a second modification is needed. For H-Ras (red) and N-Ras (orange) this is palmitoylation of one (N-Ras) or two (H-Ras) cysteines carried out by the palmitoyltransferase DHHC9 (blue). In K-Ras (purple) the need for the second modification is replaced by a string of six lysines that confer a positive charge for interaction with lipids. The dynamic re-localization of Ras proteins is further regulated by ubiquitination, phosphorylation, and the chaperone PDEδ.